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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Our previous results have shown that the basement membrane (BM) regulated the expression and function of estrogen receptor-alpha (ERα) in mouse mammary epithelial cells. New results shown here indicate that the presence of lactogenic hormones was required for the regulatory effect of BM on ERα levels. We present evidence that cell adhesion to the BM components collagen-IV, through α2 and β1 integrin subunits and laminin-1, through α2, α6 and β1 subunits are the relevant interactions responsible for transducing the signal of the BM that increases ERα expression. On the other hand, BM- induced changes in cell proliferation and cell morphology were not involved. Thus, the changes observed in ER expression and estrogenic effect when mammary epithelial cells are removed from the gland and placed in culture could be due to the disruption of the tissue organization and, in particular, to the lack of cell-matrix interactions on tissue culture plastic. Our system model could be useful to better understand the mechanisms involved in the regulation of ER expression and function during mammary gland development and breast tumor progression.

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Introduction:

When rodent or human primary mammary epithelial cells are placed in monolayer cultures, they rapidly lose their functional and morphological characteristics, including steroid hormone receptor expression, such as ER (reviewed in Ronnov-Jessen 1996; Anderson 1998). It has been proposed that the expression and function of ER depend on the cellular context. For example, isolated mammary epithelial cells in culture recover growth in response to estrogen when they are co-cultured with stromal cells (Haslam 1986, reviewed in Haslam 2001), suggesting that the specialized form of extracellular matrix (ECM) termed basement membrane (BM) produced by stromal cells can regulate epithelial cell function. In this regard, it has been shown that the expression of ERα is maintained at higher levels in mouse and human mammary epithelial cells cultured in three dimensional collagen I gels (Edery 1985; Yang 2000). However, under these conditions primary cultures might produce their own BM (Streuli 1990) and thus obscure the analysis. Thereby the involvement of particular components and effects of the BM on ER expression should be analyzed in detail.

Body:

One of our tasks in the proposal was to study transcriptional and translational modifications of ERα elicited by ECM. The fact that ERα mRNA levels were increased in the presence of BM (previous results) suggests that a transcriptional regulation of the steroid receptor could be involved. To continue with this study and to follow another task of our proposal, the characterization of DNA elements present in the ERα promoter that might responsible for the BM-effect, we are now using the following approach: we are transfecting the mammary epithelial cell line (Scp2) with plasmids that contain the β-galactosidase reporter gene under the control of DNA fragments of varying lengths corresponding to the ERα promoter, up to 5 kb upstream of the transcription start site of the ERα gene. This constructs were provided by Cicatiello *et al.* (1995) who used them to identify a 0.4 kb region of the ERα promoter that was responsible for the expression of the gene in mouse tissues.

The task regarding the physiological significance of the ERα regulation by BM was completed, since we have developed a culture system that consists of the addition of BM components, specifically collagen-IV or laminin-1, which was effective in maintaining the expression of ERα levels to approximately 50% of its level in isolated organoids (previous results). However, we have discovered that lactogenic hormones, hydrocortisone and prolactin had to be present in the medium in order to allow the BM effect on ERα levels (Figure 1).

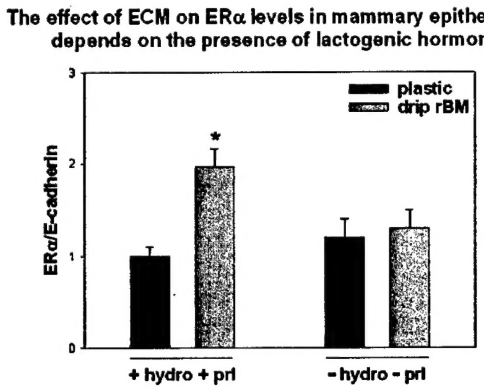
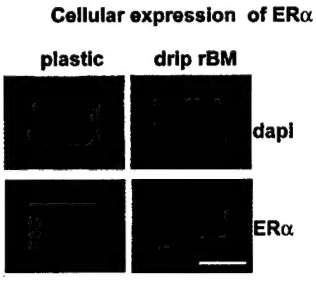


*Figure 1. Quantitative analysis of ERα protein levels determined by western blots (antibody MC-20 Santa Cruz) in lysates of mouse mammary primary epithelial cells (isolated from mammary glands of normal nulliparous BALB/c mice) cultured for 2days in the presence or absence of hydrocortisone and prolactin (1 μg/ml and 3 μg/ml, respectively). The cultures were performed on plastic or in the presence of rBM. *:p<0.05 vs. plastic.*

In order to dissect the mechanisms by which BM regulates ERα expression we used a normal mouse mammary epithelial cell line established in our laboratory wherein we have previously shown (preliminary results) that BM can induce ERα expression. This study resulted in the following results:

<u>rBM increases the number of ERα-expressing cells</u>

The effect of a reconstituted basement membrane (rBM, Matrigel) on ERα levels in mammary epithelial cells could be the consequence of increased levels of ERα expression in every cell or an increase in the fraction of cells expressing ERα. To distinguish between these possibilities, we determined by immunocytochemistry the percentage of Scp2 cells expressing ERα and the level of expression per cell, when Scp2 cells were cultured in the presence or absence of BM. We found that dripping rBM on the cells increased the percentage of ERα-expressing cells from 21% (on plastic) to 53% (drip rBM) (Figure 2). However the intensity of fluorescence per cell (ERα level/cell) was not significantly affected by rBM. These data suggest that rBM can maintain or even increase the population of ER-positive cells.



	plastic	drip rBM
% ERα-positive cells	21 +/- 4	53 +/- 3
ERα level/cell (A.U.)	119+/-14.5	101+/-9.5

Figure 2. Immunocytochemistry of Scp2 cells fixed with –20°C methanol:acetone (1:1) and incubated with ERα monoclonal antibody (NCL-ER-6F11) followed by FITC-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA), mounted and observed under fluorescence microscopy. Before mounting, 4',6-Diamidino-2-Phenylindole (DAPI, Sigma) was used to stain DNA. Images were captured using Spot RT camera and software (Technical Instruments, Burlingame, CA). Note that not all the cells (DAPI nuclear; blue) stained for ERα (green), and that the staining for ERα was predominantly nuclear. Magnification 60x. (bar, 20 μm). The table shows mean +/- standard deviation corresponding to percentages of ERα-positive cells and to ERα expression levels quantified in single cells grown on plastic or in the presence of rBM. Intensity of fluorescence was expressed as arbitrary units (A.U.). To determine cellular labeling indices for ERα we counted at least 100 cells from randomly selected visual fields and calculating the intensity of labeling in the cells by using Simple PCI imaging software (Compix, Inc. Imaging Systems, Cranberry Township, PA).

The effect of BM on ERα increase is not due to changes in cell proliferation

Steroid receptor expression in epithelial cells could be negatively affected by the proliferation status of the cells (Shoker 1999) and rBM inhibits growth of cultured mammary epithelial cells (Petersen 1992). Therefore, it was necessary to determine if changes in cell proliferation induced by rBM might be responsible for the increased ERα levels. We cultured Scp2 cells in the presence of different concentrations of insulin, which alters the rate of their proliferation. Proliferation of Scp2 cells, as measured by BrdU incorporation, was strongly stimulated by insulin in cells cultured on plastic (Figure 3 A), but as expected this hormone had much less stimulatory activity when cells were cultured in the presence of rBM (Figure 3 A). Analysis of ERα under these different culture conditions showed that its expression levels did not correlate with the proliferation status of the cells, neither on plastic nor in the presence of rBM (Figure 3 B). These results indicate that cell proliferation and ERα expression are not correlated in our system, and that the increase in ERα protein levels after addition of rBM is not due to reduced cell proliferation.

The regulatory effect of BM on ERα levels is not due to changes in growth rate

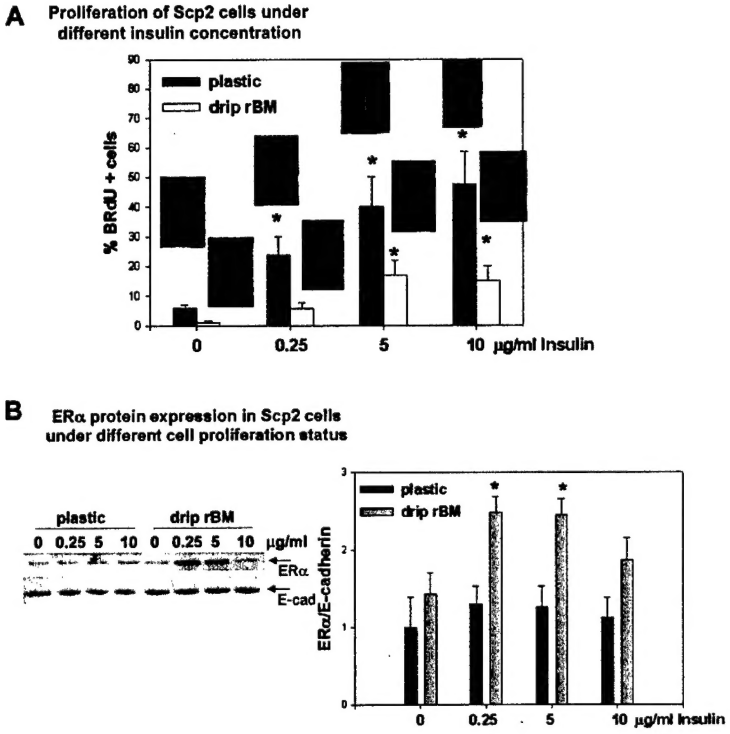


*Figure 3. **A.** Proliferation of Scp2 cells grown on plastic or in the presence of rBM after adding different concentrations of insulin (for 24 hs, in the absence of serum or other growth factor). Proliferation was determined by BrdU labeling followed by immunofluorescence. Cells that are positive for BrdU appear cyan after overlying with the nuclear staining performed with DAPI (blue). Nuclear labeling indices were determined by counting at least 100 cells from randomly selected visual fields and calculating the percentage of cells with labeled nuclei. Magnification 20x. **B.** ERα protein levels in Scp2 cells grown under different cell proliferation status indicated in A were evaluated by western blot (a representative gel is shown to the left) and quantified by densitometry (right). Replicates n=4. *:p<0.05 vs. no insulin.*

The effect of BM on ERα regulation is independent of cell shape changes

Another effect of adding rBM to epithelial cells is the change from flattened to rounded cell morphology. To find out if cell rounding per se is responsible for the regulatory effect on ERα expression, we cultured primary mammary epithelial cells and Scp2 cells on polyHEMA-coated dishes, an inert substratum which prevents cell attachment and forces the cells to remain in suspension as rounded-aggregates (Figure 4). Adding rBM to mammary epithelial cells grown on polyHEMA did not affect the cell rounding (Figure 4). However, cell rounding per se did not alter ERα protein levels in either primary cells at 2 and 6 days in culture or in Scp2 cells after 4 days in culture (Figure 4 graphs). This observation indicates that morphological changes per se are not necessary for the regulation of ERα levels by ECM. The presence of rBM in the culture medium was required for up-regulation of ERα protein levels in both primary cells and Scp2 cells.

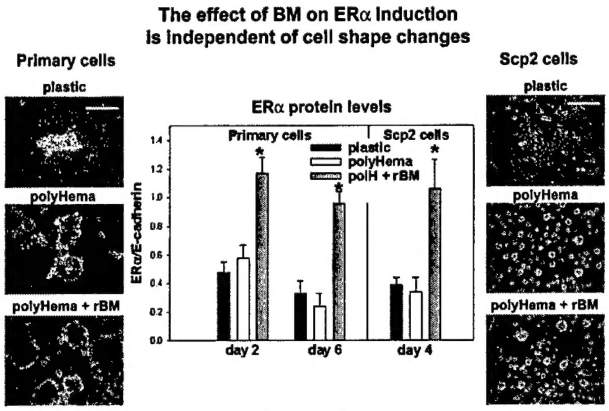


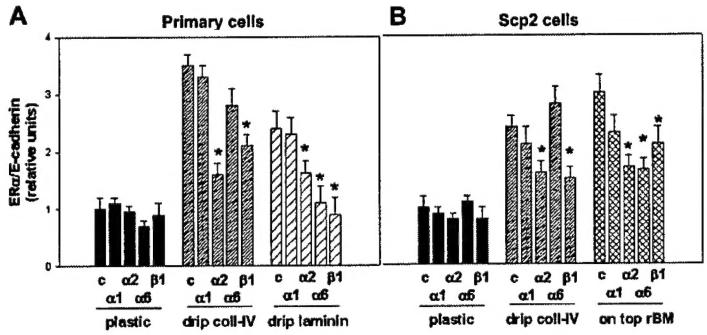
*Figure 4. Micrographs show primary cells (left) and Scp2 cells (right) forming typical flattened monolayers on plastic. Morphological change (cell rounding and aggregation) can be induced by the non-adherent substratum polyHEMA in primary cells and in Scp2 cells. The addition of rBM on polyHEMA did not affect this cell rounding. Graph bars show the densitometric analysis of ERα protein levels determined by western blot in primary mammary epithelial cells at days 2 or 6 after isolation and in Scp2 cells at day 4 in culture. The addition of rBM to pre-rounded cells was necessary to up-regulate ERα. Replicates n=3. *:p<0.05 vs. plastic. Magnification 10x (bar 100 μm).*

<u>The regulatory effect of BM components, collagen-IV and laminin, on ERα expression can be abrogated by antibodies directed against specific integrin subunits.</u>

The fact that individual BM components could reproduce the regulatory effect of rBM on ERα expression (preliminary results) motivated us to use specific integrin blocking antibodies to determine the level at which rBM exert this effect in mammary epithelial cells. Mammary epithelial cells express α1β1, α2β1, α3β1, α6β1 and α6β4 heterodimers in vivo, all of which bind to laminin (reviewed in Alford 1996 and Mercurio 2001), although α1β1 and α2β1 mainly serve as collagen receptors (Zutter 1990). We analyzed the effect of blocking antibodies directed specifically against -α1, -α2, -α6, or -β1 integrin subunits. These antibodies did not affect cell viability as determined by the Alamar blue assay (data not shown). Primary mammary cells cultured on plastic, or in the presence of collagen-IV or laminin-1, and Scp2 cells cultured on plastic, or in the presence of collagen-IV or rBM were treated with specific integrin blocking antibodies. In both, primaries and Scp2 cells, α2, α6, and β1 antibodies partially blocked the effect of rBM on ERα protein levels (Figure 5 A). Specifically, α2 and β1 antibodies blocked the regulatory effect of collagen-IV on ERα protein levels, whereas α2, α6, and β1 antibodies effectively abolished the regulatory effect of laminin-1. In contrast, α1 blocking antibody did not significantly interfere with the effects of rBM, collagen-IV or laminin-1. It is interesting to mention that there was no evident alteration in the induction of cell shape changes by rBM in Scp2 and primary cells in the presence of integrin blocking antibodies (Figure 5 B shows primary mammary epithelial cells in the presence of α6 blocking antibody). These results indicate that integrin-mediated cell adhesion to

BM components and the consequent activation of integrin signaling pathways are responsible, at least in part, for the regulation of ERα levels in mammary epithelial cells.

Collagen-IV through α2 and β1, and laminin-1 through α2, α6 and β1 integrin subunits regulate ERα levels in mammary epithelial cells



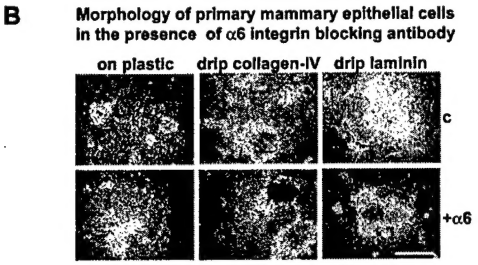


*Figure 5. ERα protein levels were determined by western blot in primary mammary epithelial cells and Scp2 cells after adding mouse IgG (control, c), or 10 μg/ml of α1, α2 or α6 or 5 μg/ml of β1 integrin blocking antibodies to cells grown for 4 days on plastic or in the presence of collagen-IV, laminin-1 or rBM. Replicates=3. *:p<0.05 vs. control (IgG addition).*

Key research accomplishments:

- **The regulatory effect of BM on ERα expression even though it did not involve functional differentiation of mammary epithelial cells (previous results showed that collagen-IV can induce ERα expression without inducing β-casein expression), required the presence of lactogenic hormones hydrocortisone and prolactin.**

- **The effect of BM on ERα expression was independent of its effects on epithelial cell morphology and cell proliferation.**

- **The expression of ERα in normal mouse mammary epithelial cells was up-regulated by the presence of cell-BM interactions. In particular, cell interactions with collagen-IV and laminin-1.**

- **The effect of collagen-IV on ERα expression involved the α2β1 integrin heterodimer, whereas the effect of laminin-1 involved α2β1 and α6β1 heterodimers.**

Reportable outcomes:

A publication in progress (unpublished) is provided with this report (Appendix). It will be submitted soon to Molecular Biology of the Cell. The manuscript includes preliminary results obtained under a previous DOD-BCRP fellowship DAMD 17-97-1-7239 and the present results obtained under the fellowship DAMD 17-01-1-0293.

These results were presented or will be presented in the following meetings:

- 6/8 - 6/11/2000: Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Atlanta, Georgia, USA. "Extracellular matrix regulation of estrogen receptors in mouse mammary cells". V.Novaro, C.Roskelley and M.J.Bissell.
- 4/6 - 4/10/2002: American Association for Cancer Research, 93rd Annual Meeting, San Francisco, CA, USA. "Estrogen receptor expression and function are regulated by the extracellular matrix in mouse mammary cells". V.Novaro and M.J.Bissell.
- 6/1 - 6/4/2002 (originally programmed for September 29- October 2, 2001): Reaching for the Cure...Innovations in Quality Care, The Susan G. Komen Breast Cancer Foundation, Washington, D.C. USA. "Extracellular matrix regulation of estrogen receptors in mouse mammary epithelial cells". V.Novaro and M.J.Bissell.
- 10/25 – 10/28/2002: Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Orlando, Florida, USA. "Extracellular matrix regulation of estrogen receptors in mouse mammary epithelial cells". V.Novaro and M.J.Bissell.
- 12/14 – 12/18/2002: 42nd Annual Meeting of the American Society for Cell Biology, San Francisco, CA, USA. "Cell adhesion to collagen-IV or laminin-1 modulates estrogen receptor-alpha (ERα) expression in mouse mammary epithelial cells". V.Novaro, C.D.Roskelley and M.J.Bissell.

Conclusions:

We have determined that cell adhesion to collagen-IV or laminin-1 through particular integrins up-regulated ERα levels both in primary mammary epithelial cells and in an established functionally normal mammary epithelial cell line. On the other hand, even though the presence of rBM diminished the growth rate and induced cell rounding, these two phenomena were not responsible of the regulatory effect observed on ERα expression in mammary epithelial cells. Thus, the lack of cell-matrix interactions

when epithelial cells were placed on tissue culture plastic appeared to be responsible for the deregulation in their estrogenic response. Furthermore, our data provide a possible explanation for the loss of ER expression that occurs during breast tumor progression: as mammary epithelial cells progress to malignancy and invasion, they lose normal interactive contact with the BM (Petersen 1992; Werb 1996; Bissell and Radisky 2001), thereby losing the signals that maintain the normal expression of ER. Therefore, our culture model system may be used to understand why some breast cancers develop as ER-positive tumors, and some develop as more aggressive hormonal-resistant ER-negative ones.

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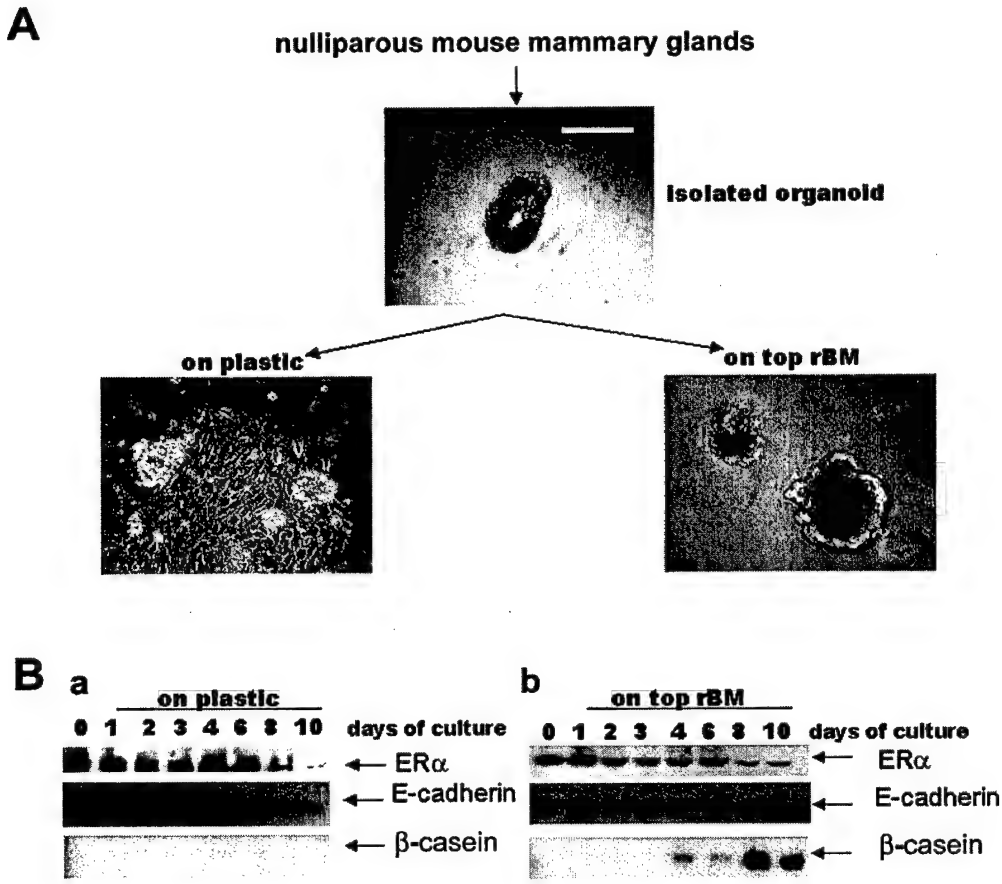
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ERα/E-cadherin (culture days/day 0)
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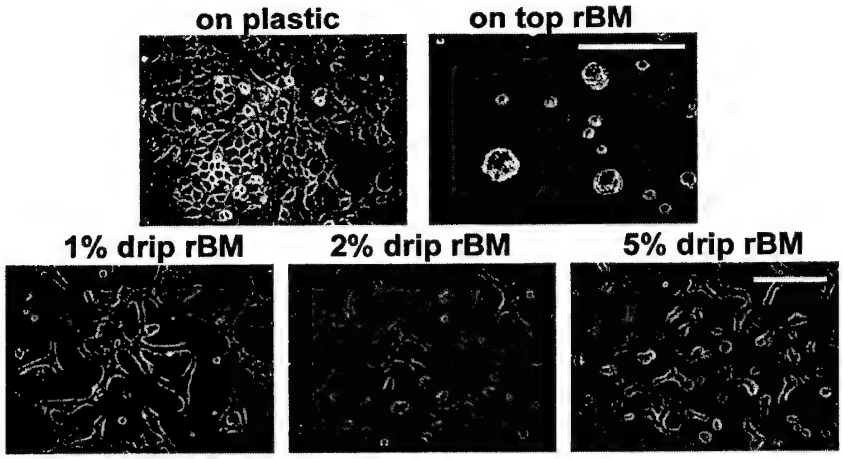
A



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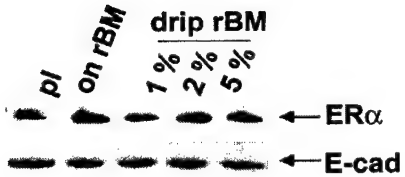

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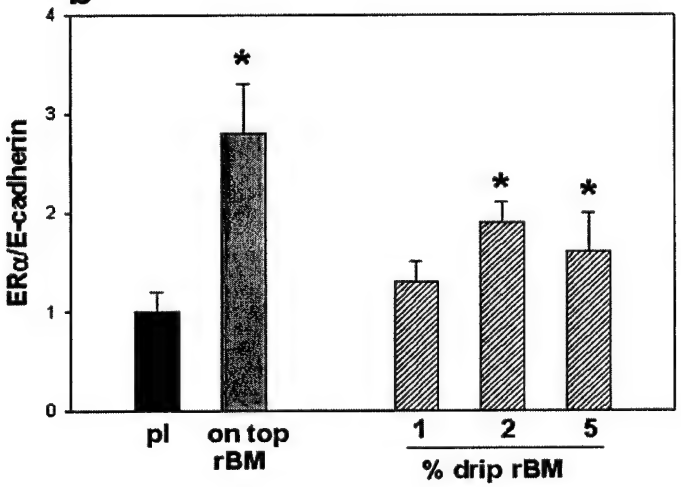

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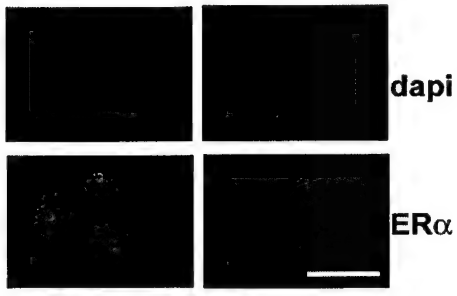
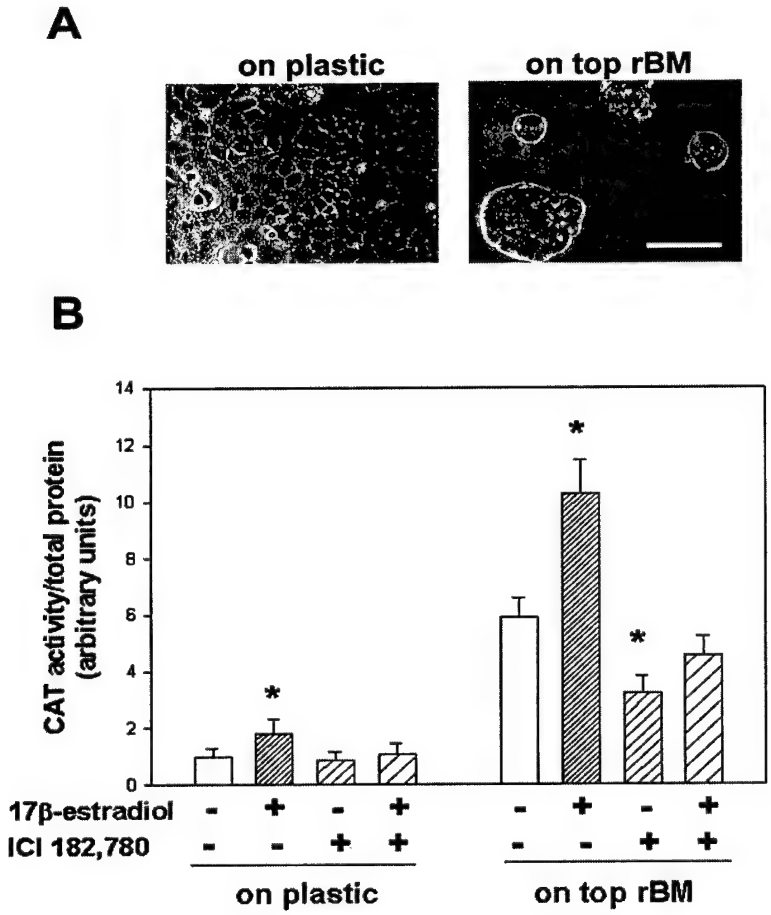


b

	plastic	drip rBM
% ERα-positive cells	21 +/- 4	53 +/- 3
ERα level/cell (A.U.)	119+/-14.5	101+/-9.5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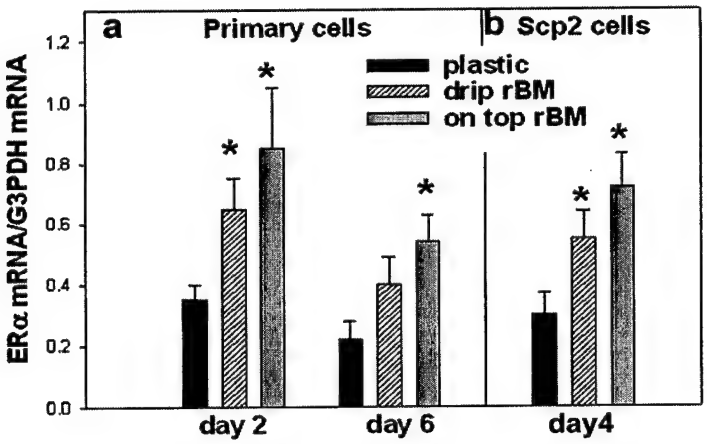
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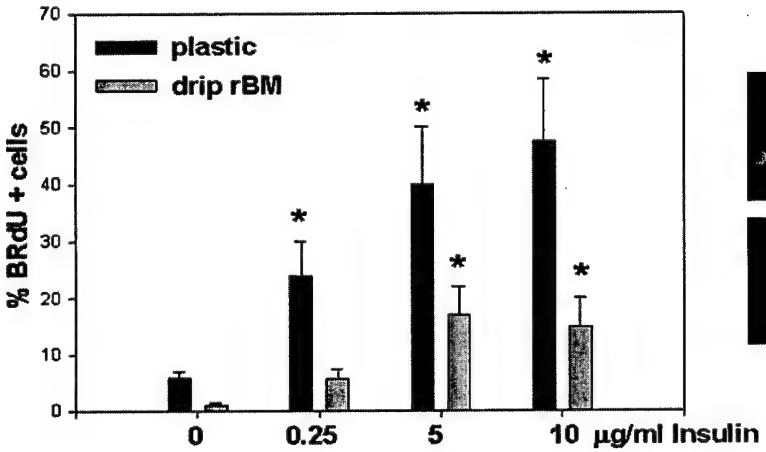
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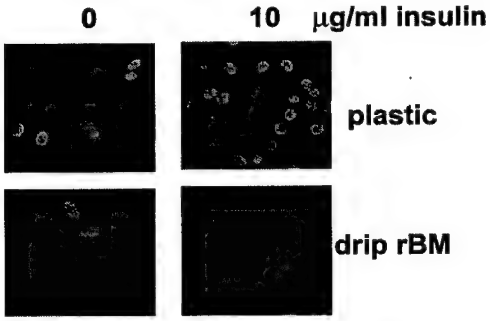
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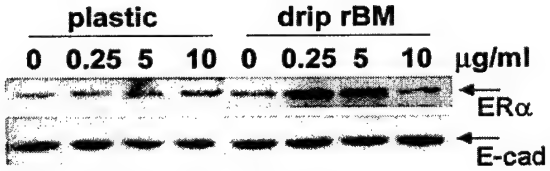
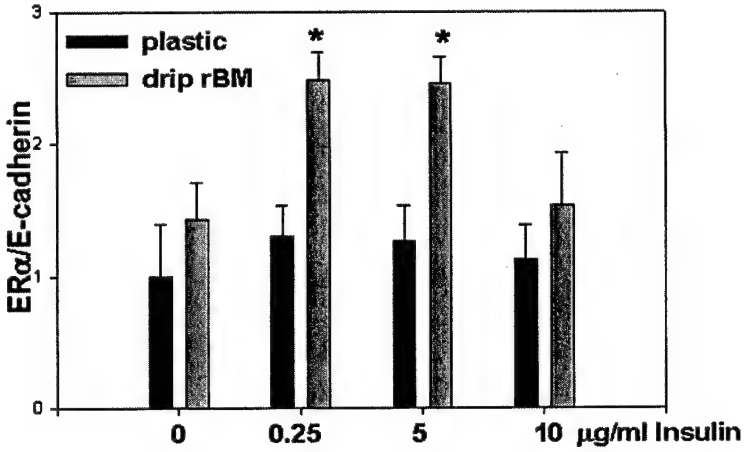
B

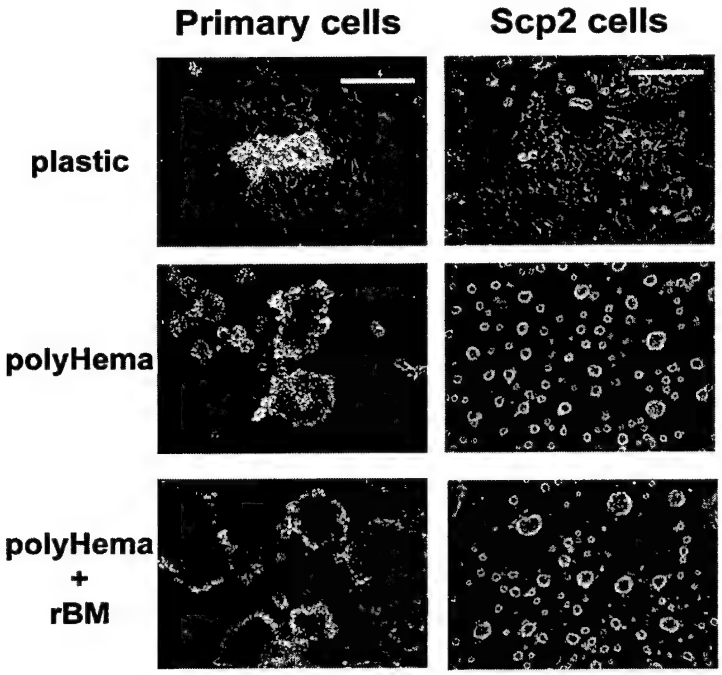
a


plastic
drip rBM
0 0.25 5 10 0 0.25 5 10 µg/ml
ERα
E-cad

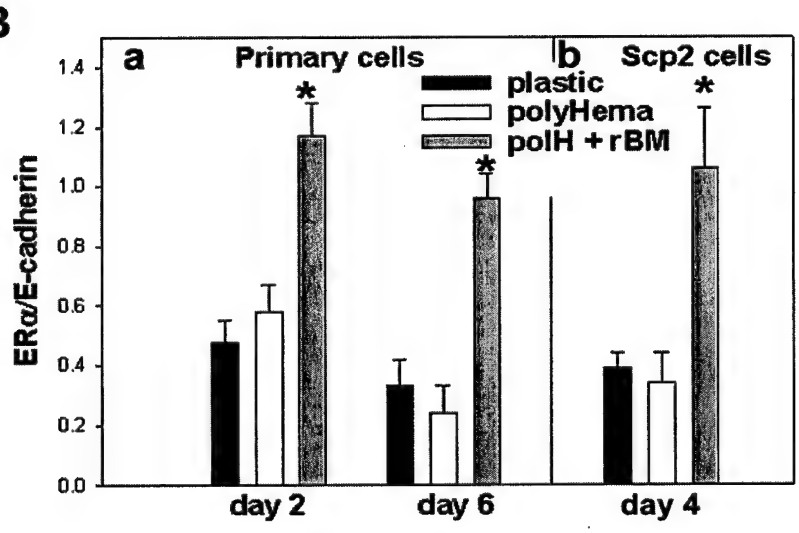
b

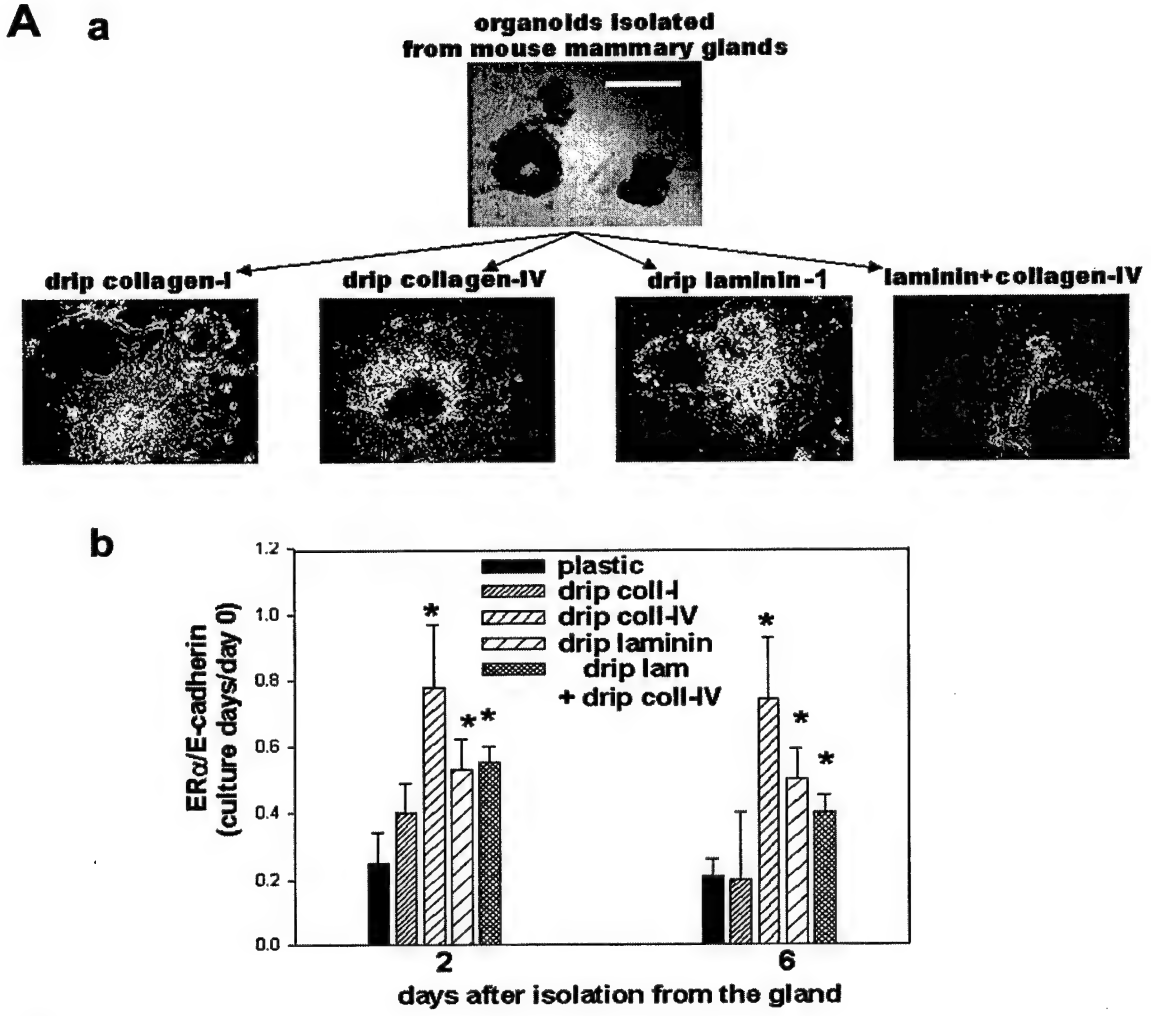

plastic
drip rBM
ERα/E-cadherin
3
2
1
0
*
0
0.25
5
10 µg/ml Insulin

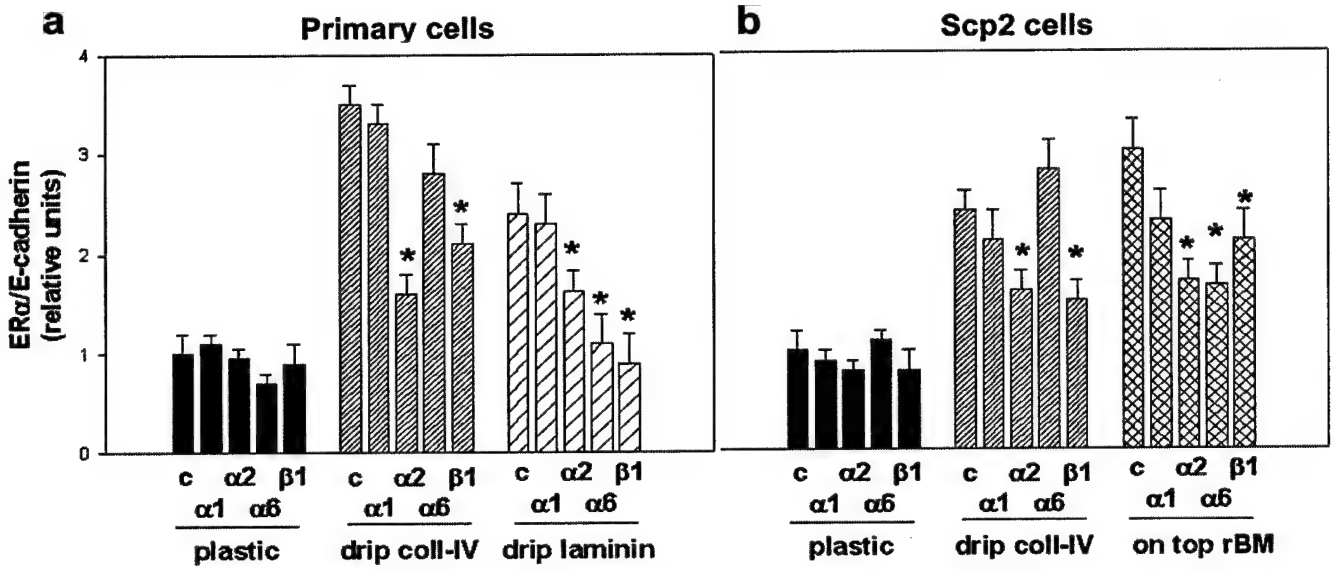
A


Primary cells
Scp2 cells
plastic
polyHema
polyHema
+
rBM

B


a
Primary cells
b
Scp2 cells
plastic
polyHema
polH + rBM
ERα/E-cadherin
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
day 2
day 6
day 4


A
a
organoids isolated
from mouse mammary glands
drip collagen-I
drip collagen-IV
drip laminin-1
laminin+collagen-IV
b
plastic
drip coll-I
drip coll-IV
drip laminin
drip lam
+ drip coll-IV
ERα/E-cadherin
(culture days/day 0)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2
6
days after isolation from the gland
B
a
drip collagen-I
drip collagen-IV
drip laminin-1
b
ERα/E-cadherin
3
2
1
0
pl
coll-I
coll-IV
lam
drip


a
Primary cells
b
Scp2 cells
ERα/E-cadherin
(relative units)
0
1
2
3
4
c
α2
β1
α1
α6
plastic
drip coll-IV
drip laminin
on top rBM

Collagen-IV and laminin-1 regulate estrogen receptor-alpha expression and function in mouse mammary epithelial cells.

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Abstract

The expression level of estrogen receptor alpha (ERα) is an important determinant of breast function as well as breast cancer prognosis. However, how its level is regulated in vivo is poorly understood. Whereas the expression of ERα is greatly reduced when primary mammary epithelial cells are placed in culture, we found that its expression level in primary mammary fibroblasts is not altered, indicating that this effect is cell type-specific. We established a culture system where a reconstituted basement membrane (rBM) partially prevented the decrease of ERα levels in primary mouse mammary epithelial cells without affecting its levels in primary fibroblasts. Furthermore, rBM increased ERα expression three-fold and ER-dependent reporter gene expression six-fold in a functionally normal mammary epithelial cell line. The regulatory effect of rBM on ERα was reproduced by two of its components, collagen-IV and laminin-1, and it was blocked by antibodies against integrin subunits α2, α6, and β1. Our results indicate that integrin-mediated cell adhesion to particular basement membrane components, more than cell rounding or cell growth arrest induced by rBM, is critical in the regulation of ERα expression and its transcriptional activity. Thus, maintenance of tissue organization and the microenvironmental context are important determinants of proper estrogenic function in the mammary gland.

Key words

Basement membrane, mammary fibroblasts, cell-extracellular matrix interactions, integrins, cell shape changes

Running title

ERα regulation by basement membrane

Introduction

Estrogen controls many aspects of cell proliferation and differentiation in the mammary gland through its two homologous intracellular receptors, estrogen receptor α and β (ERα and ERβ) (reviewed in {Gustafsson 2000; Cunha 2000}). ERα is expressed at low levels in the normal mammary gland and its deregulation is strongly correlated with the progression of mammary neoplasia to invasive and ductal carcinomas {Sommer and Fuqua 2001}. In the mammary gland, ERα and ERβ act as hormone-dependent transcriptional regulators of several genes involved in cell cycle control and mammary gland development, including the progesterone receptor (PR) responsible of most of the mammary gland growth {Kastner 1990}. Although estrogen regulates gene expression and cell proliferation in vivo, normal mammary epithelial cells, unlike many ER-positive breast cancer cells, do not proliferate in response to estrogen when cultured as monolayers {Edery 1984}. Furthermore, when rodent or human primary mammary epithelial cells are placed in monolayer cultures, they rapidly lose their functional and morphological characteristics, including steroid hormone receptor expression, such as ER (reviewed in {Ronnov-Jessen 1996; Anderson 1998}). It has been proposed that the expression and function of ER depend on the cellular context as well as on the availability of receptor interacting proteins, co-activators and co-repressors {White 1998}. For example, isolated mammary epithelial cells in culture recover growth in response to estrogen when they are co-cultured with stromal cells {Haslam 1986, reviewed in Haslam 2001}, suggesting that the specialized form of extracellular matrix (ECM) termed basement membrane (BM) produced by stromal cells can regulate epithelial cell function. In this regard, it has been shown that the expression of ERα is maintained at higher levels in mouse and human mammary epithelial cells cultured in three dimensional collagen I gels {Edery 1985; Yang 2000}. However, under these conditions primary cultures might produce their own BM {Streuli 1990} and thus obscure the analysis. Thereby the involvement of particular components and effects of the BM on ER expression should be analyzed in detail.

The BM in vivo surrounds mammary epithelial cells and separates them from the underlying stroma. It is rich in laminin-1 and it also contains collagen-IV, entactin, proteoglycans and other glycoproteins (reviewed in {Aumailley 1998}). Signals from the BM regulate cell structure, polarization, growth, functional differentiation and the rate of apoptosis in epithelial mammary cells {Stoker 1990; Boudreau 1998; Bissell 1999}. When mammary epithelial cells are cultured in the presence of a reconstituted BM (rBM) or purified laminin-1, they arrest and reorganize into tissue-like structures (acini) which secrete milk proteins into a central lumen when lactogenic hormones, hydrocortisone and prolactin, are present {Barcellos-Hoff 1989}. Using a clonal mammary epithelial cell line Scp2 established in our laboratory that is unable to produce its own BM {Desprez 1993}, we previously found two distinct signals that control β-casein expression in response to laminin-1. These signals consist of a morphogenic signal that involves cell shape changes (cell rounding), and a subsequent biochemical signal that involves integrins {Roskelley 1995; Streuli 1995; Muschler 1999} the transmembrane receptors that connect the cell cytoskeleton to the ECM {Giancotti 1999}. Therefore, by using Scp2 cells, one avoids the interference from endogenous BM deposition, a problem that occurs with heterogeneous cell cultures, such as primary cultures where the effect of some remaining myoepithelial and fibroblast cells which secrete ECM components, can not be ruled out {Muschler 1999}.

Tissue-specific functions related to both cell morphology and gene expression are regulated by signals from the microenvironment {Bissell 1999}. In particular, cell contact with the BM constitutes a critical regulator of cell structure and function. We hypothesized that the deregulation of ER expression in mammary cells cultured on tissue culture plastic occurs as the result of the lack of appropriate cell-matrix interactions. Inasmuch as disruption of cell-ECM interactions is a hallmark of breast and other forms of cancer {Bissell and Radisky 2001}, our results may provide insight into the progression towards hormone-independent tumors and the consequent resistance to endocrine therapies developed in certain types of invasive breast carcinomas.

Results

A reconstituted basement membrane (rBM) prevents the decrease in ERα expression by primary mammary epithelial cells placed in culture. ERα expression in primary fibroblasts is independent of rBM.

To test whether the loss of ERα expression in culture is due to disruption of endogenous BM and to determine the kinetics of ERα expression in freshly isolated cells, we placed mammary epithelial organoids (Figure 1 A a) isolated from nulliparous mammary glands in culture in the presence or absence of rBM (Figure 1 A b,c). Twenty-four hours after plating, almost 70% of the organoids had attached and spread on plastic, and by 2-3 days of culture almost all of them had formed monolayers (Figure 1 A b). We found a significant reduction of ERα protein levels in epithelial cells cultured on tissue culture plastic even one day after isolation (Figure 1 B a), and we observed a further steady decrease throughout the rest of the culture period (nine days). However, when the organoids were cultured on top of rBM, their morphology remained essentially intact until the end of the culture period (day 10) (Figure 1 A c) and the initial drop in ERα levels was significantly less pronounced than cells on plastic (Figure 1 B b), consequently, ERα levels remained significantly higher than on plastic throughout the 10 day culture period (Figure 1 B a,b,c). We also measured β-casein synthesis in primary epithelial cells derived from nulliparous mammary glands. As expected, primary epithelial cells grown on plastic did not synthesize β-casein (Figure 1 B -a). Detectable expression of this milk protein was found after 4 days in culture in the presence of rBM and lactogenic hormones (Figure 1 B -b), and its expression continued to increase throughout the end of the culture period.

It is conceivable that ERα positive cells attach less strongly to tissue culture plastic than ERα negative cells or that the lower levels of ERα are caused by cell spreading. This would produce an enrichment of ERα negative cells in cells cultured on plastic. To determine if ERα expressing cells attached differentially to plastic, we measured ERα protein levels in cells and organoids that did or did not attach to tissue culture plastic during the first 24 hours of culture. ERα protein level in adherent cells was similar to that of the cell that had attached and spread (data not shown).

In vivo, ERα is expressed in a fraction of the mouse mammary epithelial and stromal cells {Shyamala 2002}. To determine if ER expression in mammary stromal cells is also under the control of BM, we measured ERα levels in primary mammary fibroblasts immediately after isolation (time 0), and after 2 and 6 days of culture in the presence or absence of rBM. Primary fibroblasts cultured on plastic adopted typical spindle-shape morphology, whereas on top of rBM they formed aggregates (Figure 1 C). At the beginning of the time course (data not shown) as well as by day 2 in culture (Figure 1 D) ERα levels in fibroblasts were slightly lower than those in epithelial cells. However, in contrast to the reduction of ERα expression observed in epithelial cells, ERα levels in fibroblasts did not change during the course of the culture (data not shown) and were not affected by the presence of rBM (Figure 1 D). This result implies that unlike epithelial cells, the ERα level in primary fibroblasts is not regulated by ECM.

Using a functional mammary cell line to dissect the BM response

To dissect the molecular mechanisms involved in the response of mammary epithelial cells to BM and to determine if BM also regulates the de novo synthesis of ERα , we utilized a clonal mouse mammary epithelial cell line, Scp2 {Desprez 1993}, that does not deposit an endogenous BM. These cells require the addition of an exogenous BM to functionally differentiate in the presence of lactogenic hormones.

Scp2 cells were cultured a- on plastic, where they adopted a flattened morphology (Figure 2 A a), b- on top of rBM, where they formed acinus-like structures (Figure 2 A b), or c- cultured on plastic with different dilutions of rBM added to the medium (Figure 2 A c,d,e), using a previously established procedure {Roskelley 1994}. For this last condition, the cells were grown on plastic and after attachment they were overlaid with the rBM diluted in the incubation medium in different concentrations. We found

that this procedure, we refer to as "dripping", allows us to analyze individual ECM components (see below).

We measured ERα protein levels in Scp2 cells cultured in the presence or absence of rBM and found that the ERα levels were up-regulated two-three-folds by rBM (Figure 2 B a,b). Dripped rBM, while not as effective as a thick gel, nevertheless induced ERα significantly. It is noteworthy that 2% dripped rBM was sufficient to up-regulate ERα levels in Scp2 cells. These experiments suggested that the effect of rBM on ERα expression was not directly related to the effect of rBM on cellular morphology, since 5% rBM had no additive effect in regulating ERα levels in spite of its larger effect on cell morphology (Figure 2 A c,d,e).

The effect of rBM on ER levels could be the consequence of increased levels of ERα expression in every cell or an increase in the fraction of cells expressing ERα. To distinguish between these possibilities, we determined by immunocytochemistry the percentage of Scp2 cells expressing ERα and the level of expression per cell, when Scp2 cells were cultured in the presence or absence of BM. We found that dripping rBM on the cells increased the percentage of ERα-expressing cells from 21% (on plastic) (Figure 2 C a,b) to 53% (drip rBM) (Figure 2 C c,d,e). However the intensity of fluorescence per cell (ERα level/cell) was not significantly affected by rBM (Figure 2 C f). These data suggest that rBM can maintain or even increase the population of ER-positive cells.

Mouse mammary epithelial cells respond to BM by up-regulating ER-mediated transcriptional activity.

The estrogenic effect involves the binding of dimers of ER to a target gene promoter that contains a palindromic estrogen response element (ERE). To determine if the regulation of ERα levels is reflected in an increase in the activity of the receptor, we transfected Scp2 cells with an ERE-response element attached to a reporter gene, generating a functionally normal Scp2 ERE-TK-CAT cell line. These cells, cultured on top of rBM, underwent the characteristic morphological differentiation (Figure 3 A a,b) observed in the non-transfected Scp2 cells (Figure 2 A a,b) i.e. they resembled mammary organoids in culture (Figure 1 A b,c), and in the presence of lactogenic hormones they functional differentiated and produced β-casein (data not shown). Under these conditions, the cells exhibited a six-fold higher CAT activity than the cells cultured on plastic (Figure 3 B). However, the effect of rBM on CAT activity was independent of the presence of 17β-estradiol (Figure 3 B). When estradiol was added to the medium we did not detect any morphological effect (data not shown) and the folds increase in reporter activity was similar on both plastic and rBM. The addition of the ER antagonist, ICI-182,780 blocked the increase in CAT activity induced by estradiol but only partially blocked the increase induced by rBM. Taken together, these results suggest that rBM stimulates a ligand-independent activity of ER in mouse mammary epithelial cells.

ERα mRNA levels are regulated by BM in both primary mammary cells and the clonal epithelial cell line

To determine if BM regulates ERα expression at the transcriptional level, we measured ERα mRNA levels by quantitative RT-PCR. We found that in primary mammary epithelial cells, ERα mRNA decreased during the initial 24 hours culture period, whether the cells were cultured in the presence or absence of rBM (data not shown). This decrease is similar to the decrease of ERα protein levels found in the same cells (Figure 1 B c). After 2 and 6 days in culture, ERα mRNA levels were significantly higher when the organoids were cultured on top of rBM or after dripping rBM, relative to organoids cultured on plastic (Figure 4 A). Similarly, when ERα mRNA levels were evaluated in Scp2 cells, we found a two-three-fold increase when the cells were growing for 4 days in the presence of rBM compared to cells grown on plastic (Figure 4 B). These results indicate that the regulatory effect of rBM on ERα expression in mammary epithelial cells is exerted, at least in part, at the mRNA level. It is noteworthy that the relative levels of ERα mRNA are similar in primaries and the cell line.

The effect of BM on ERα increase is not due to changes in cell proliferation

Steroid receptor expression in epithelial cells could be negatively affected by the proliferation status of the cells {Shoker 1999} and rBM inhibits growth of cultured mammary epithelial cells {Petersen 1992}. Therefore, it was necessary to determine if changes in cell proliferation induced by rBM might be responsible for the increased ERα levels. We cultured Scp2 cells in the presence of different concentrations of insulin, which alters the rate of their proliferation as shown previously in our laboratory {Srebrow 1998}. Primary mammary cells could not be used for this experiment, since they require insulin to survive in culture. Proliferation of Scp2 cells, as measured by BrdU incorporation, was strongly stimulated by insulin in cells cultured on plastic (Figure 5 A a,b,c), but as expected this hormone had much less stimulatory activity when cells were cultured on rBM (Figure 5 A a,d,e). Analysis of ERα under these different culture conditions showed that its expression levels did not correlate with the proliferation status of the cells, neither on plastic nor in the presence of rBM (Figure 5 B a,b). These results indicate that cell proliferation and ERα expression are not correlated in our system, and that the increase in ERα protein levels after addition of rBM is not due to reduced cell proliferation.

The effect of BM on ERα regulation is independent of cell shape changes

Another effect of adding rBM to epithelial cells is the change from flattened to rounded cell morphology (Figure 1 A and 2 A). To find out if cell rounding per se is responsible for the regulatory effect on ERα expression, we cultured primary mammary epithelial cells and Scp2 cells on polyHEMA-coated dishes, which prevents cell attachment and forces the cells to remain in suspension as rounded-aggregates (Figure 6 A a-d). This type of culture reproduces many of the morphological changes caused by growth on ECM {Roskelley 1994; Muschler 1999}. Adding rBM to mammary epithelial cells grown on polyHEMA did not affect the cell rounding (Figure 6 A e,f). However, cell rounding per se did not alter ERα protein levels in either primary cells at 2 and 6 days in culture (Figure 6 B a) or in Scp2 cells after 4 days in culture (Figure 6 B b). This observation indicates that morphological changes per se are not necessary for the regulation of ERα levels by ECM. The presence of rBM in the culture medium was required for up-regulation of ERα protein levels in both primary cells and Scp2 cells.

Collagen-IV and laminin-1 are the BM components responsible for the regulation of ERα levels

To establish which components of the rBM were responsible for the enhanced maintenance of ERα in epithelial cells in culture, we dripped purified ECM components at a final concentration equivalent to their concentration in 2% rBM (see Materials and Methods). Isolated mammary epithelial organoids (Figure 7 A a) were attached and spread after 1 day in culture and the presence of ECM components did not affect this behavior (Figure 7 A b,c,d,e). Whereas collagen-I was ineffective in maintaining ERα levels, collagen-IV and laminin-1 provided partial protection against the ERα drop (Figure 7 A f). However, the combination of laminin-1 and collagen-IV did not have an additive effect, suggesting that the intracellular signals elicited by these molecules may converge on the same downstream targets.

We then evaluated the same ECM components for the up-regulation of ERα protein levels in Scp2 cells. Whereas dripping collagen -I or -IV had no evident effect on cell shape (Figure 7 B a,b), dripping laminin-1 (Figure 7 B c) or rBM (Figure 2 A c,d,e) induced cell rounding supporting the idea that laminin-1 is responsible, at least in part, of the morphological effect of rBM. As in primary cultures, collagen IV was the most effective of the ECM components tested in increasing ERα protein levels in Scp2 cells, whereas laminin-1 was effective to a lesser degree, and collagen-I or fibronectin (data not shown) had no effect (Figure 7 B d). The effect of collagen-IV on ERα levels did not involve discernable cell rounding (Figure 7 B b). This observation, together with the lack of effect of polyHEMA on the regulation of ERα, suggests that biochemical signaling by BM components was responsible for the regulation of ERα. As the effect of collagen IV and laminin-1 on ERα levels were comparable to the fold-increase effect of dripping rBM in Scp2 cells (Figure 2 B b), we concluded that the major components responsible in the matrix were collagen-IV and laminin-1.

The regulatory effect of BM components, collagen-IV and laminin, on ERα expression can be abrogated by antibodies directed against specific integrin subunits.

The fact that two individual BM components could reproduce the regulatory effect of rBM on ERα expression motivated us to use specific integrin blocking antibodies to determine the level at which rBM exert this effect in mammary epithelial cells. Mammary epithelial cells express α1β1, α2β1, α3β1, α6β1 and α6β4 heterodimers in vivo, all of which bind to laminin (reviewed in {Alford 1996 and Mercurio 2001}), although α1β1 and α2β1 mainly serve as collagen receptors {Zutter 1990}. We analyzed the effect of blocking antibodies directed specifically against -α1, -α2, -α6, or -β1 integrin subunits. These antibodies did not affect cell viability as determined by the Alamar blue assay ({Lochter 1999}, and data not shown). Primary mammary cells cultured on plastic, or in the presence of collagen-IV or laminin-1 (Figure 8 A) and Scp2 cells cultured on plastic, or in the presence of collagen-IV or rBM (Figure 8 B) were treated with the specific integrin blocking antibodies. In both cell types, α2, α6, and β1 antibodies partially blocked the effect of rBM on ERα protein levels. Specifically, α2 and β1 antibodies blocked the regulatory effect of collagen-IV on ERα protein levels, whereas α2, α6, and β1 antibodies effectively abolished the regulatory effect of laminin-1. In contrast, α1 blocking antibody did not significantly interfere with the effects of rBM, collagen-IV or laminin-1. It is interesting to mention that there was no evident alteration in the induction of cell shape changes by rBM in Scp2 and primary cells in the presence of integrin blocking antibodies (data not shown) as indicated previously by Muschler et al. {Muschler 1999/d} for α6 and β1 antibodies. These results indicate that integrin-mediated cell adhesion to BM components and the consequent activation of integrin signaling pathways are responsible, at least in part, for the regulation of ERα levels in mammary epithelial cells.

Discussion

In the current study we have found that a rBM regulates ERα expression and function in mammary epithelial cells in culture. rBM induced ERα gene expression, thereby increasing ERα protein levels and ER transcriptional activity. We found that rBM exerts its effect not by inducing changes in cell shape or cell proliferation but rather inducing cell adhesion to collagen-IV or laminin-1. When the integrity of these cell-matrix interactions was disrupted by using blocking antibodies against α2, α6 or β1 integrin subunits, the regulatory effect of rBM was abolished.

BM is a regulator of ERα expression both in cell lines and in primary cultures

We found that both immortalized non-tumorigenic Scp2 mammary epithelial cells and primary mammary epithelial cells respond to the ECM components collagen-IV and laminin with increased ERα levels. This effect seems to be largely independent of the cellular reorganization induced by rBM, since collagen-IV can reproduce the ERα-enhancing effect without bringing about the cell rounding morphology characteristic of cells cultured with whole rBM. Furthermore, culturing mammary epithelial cells on polyHema-coated dishes, which prevents cell attachment and forces the cells to remain in suspension as rounded-aggregates, does not alter ERα levels or function per se, even though it reproduces many of the morphological changes caused by growth on ECM {Roskelley 1994; Muschler 1999}. Still, Scp2 cells grown on top of rBM displayed the highest ERα levels, suggesting that cell polarity (i.e. Scp2 cells polarize when cultured on top of rBM (A. Somasiri and C.D. Roskelley, unpublished observations) might play a separate or additional role. Also, other matrix components (such as entactin or proteoglycans) should not be ruled out as potential regulators of ERα function. In the case of primary cultures, the difference in ERα expression that occurs after isolating epithelial cells from their surrounding myoepithelial and fibroblast cells suggests that other factors derived from stromal-epithelial interaction could also participate in the regulation of ERα expression.

rBM increases ERα expression in cultured mouse mammary epithelial cells

Our data suggest that the increased level of ERα in normal mammary epithelial cells in response to rBM is due, at least in part, to increased transcription of the ERα gene. Even though only a fraction of the cells had detectable levels of ERα protein by immunofluorescence, we observed that rBM rather than increase ERα expression in pre-existing ER-positive cells, increases the number of ERα-positive cells.

Previous investigations on the role of specific ECM components on ERα regulation have produced some contradictory results. MCF-7 and T47-D human tumor cell lines showed a down-regulation of ER-mediated response without affecting ER levels when cultured on laminin gels {Woodward 2000}. Our results using normal mouse mammary epithelial cells showed an up-regulatory effect of laminin-1 on ERα levels and on an ER-mediated response. These differences could be due to the fact that tumor cells do not respond to rBM as normal cells do, or due to differences between the two culture systems. On the other hand, Edery et al. {Edery 1985 /d} reported that the expression of ER was maintained by culturing mammary epithelial cells embedded within collagen I gels, while we found that ERα levels were not up-regulated by dripping collagen-I into the medium. However, primary cultures are mixed populations of epithelial and myoepithelial cells and Streuli and Bissell {Streuli and Bissell 1990/d} have shown that when they are cultured in collagen I three-dimensional gels they can synthesize and deposit their own BM. We propose that the effect of collagen I reported by Edery et al. {Edery 1985 /d} may in fact be attributed to the newly synthesized BM, rather than to the collagen I per se. We confirmed the effect of purified collagen-IV and laminin-1 in a clonal cell line that is unable to produce its own BM. This fact should pave the way for further molecular studies on the signal transduction pathway activated by BM that regulates ERα (and may be ERβ).

Hormone-independent regulation of ER activity by BM

We showed that a portion of the ECM-mediated up-regulation of ER-mediated transcriptional response was independent of the presence of estradiol, and that it was not completely blocked by the antagonist ICI182,780. Recently, several groups have reported a ligand-independent activation mechanism for ER and other steroid hormone receptors in a number of cell types and under different conditions {Kato 1998; Weigel 1998}. The activation of ER requires ER binding to the Src family of coactivators. The binding of estrogen to ER induces a conformational change that leads to the exposure of the AF2 domain, which contains the binding site for Src. In the absence of hormone, the activation of the ER could occur through a third protein that recruits the coactivators to ER {Bernards 1999}. Cyclin D1 might be one of these "bridges", since it is capable of binding ER and Src simultaneously and its overexpression induces ER-mediated responses in the absence of hormone in several cell lines, including Scp2 {Zwijsen 1997; Zwijsen 1998; Neuman 1997}. Furthermore, D type cyclins have previously been implicated as downstream targets of ECM signaling pathways {Buckley 1997; Neuman 1997; Yu 2001}. Neuman et al. {Neuman 1997/d} have shown that ECM increases cyclin D1 levels. Here, we have found that ECM stimulates an estrogen-independent ER-mediated response in Scp2 cells, and we suggest that these two ECM actions could be linked. Other evidence supporting the notion that the cellular microenvironment modulates ER function through cyclin D1 in mammary epithelial cells comes from Lamb et al. {Lamb 2000 /d}, who showed in MCF-7 cells that the association between ER and cyclin D1 is enhanced when cells are cultured in the presence of lactogenic hormones and preadipocytes. We suggest that BM may regulate ER function at multiple levels, resulting in an overall increase in ER-mediated responses in mouse mammary epithelial cells.

Hormonal status and ERα expression

We observed that the presence of lactogenic hormones (hydrocortisone and prolactin) is required for the regulatory effect of rBM on ERα levels since primary mammary epithelial cells could not maintain ERα expression in media lacking such hormones even when rBM was added (data not shown). This suggests that parallel signal transduction pathways are required to regulate ERα levels appropriately. Using medium lacking lactogenic-hormones, Xie et al. {Xie 1997 /d} reported that ER levels were not maintained in nulliparous mouse-derived cells cultured on laminin, fibronectin, collagen-I, collagen-IV or tenasin. However, ER levels were maintained when cultures were prepared from pregnant animals. Taken together with our data, these results suggest that a pretreatment of mammary epithelial cells with high prolactin levels, such as those found in pregnant animals, is necessary for the cells to be responsive in terms of ERα expression. Interestingly, certain properties of ER (acidity, molecular weight, DNA binding capacity, responsiveness to estrogen) are different in the lactating vs. non-lactating (nulliparous) mammary gland {Gaubert 1986; Shyamala 1992}, suggesting that the hormonal status of the animal during pregnancy and lactation alters ER functionality. In addition, the levels of ERα expression decrease in the mammary gland at the onset of pregnancy and then increase during lactation {Shyamala 2002}. The fact that ER regulation by ECM is different in different hormone conditions suggests that ER expression is part of the general response of the mammary gland to differentiation. Indeed, differentiation in the mammary gland includes changes in the predominant cell-type from non-epithelial to epithelial {Haslam 1981} and in the expression of different combinations of integrins {Suzuki 2000; Haslam 2001}. However, we observed that collagen-IV induced ERα expression without inducing β-casein production (data not shown) indicating that both processes, ERα and β-casein induction, are independent. We propose that changes in ERα expression are induced by cell-matrix interactions in conjunction with lactogenic hormones that then lead to changes in the estrogenic function.

The regulation of ERα by BM is mediated by integrins: relevance to tumorigenesis

We have found that integrin-activated signal transduction pathways are responsible for the adhesion-dependent effect of BM on the regulation of ERα levels. The study of integrins as ECM receptors is crucial to understanding mammary gland development and tumor progression, as alterations in the

microenvironment or altered perception of the microenvironment can cause normal cells to display tumorigenic behavior and vice versa (for review see {Bissell and Radisky 2001}). Furthermore, a reduced expression of α2-, α3-, α6-, β1- and β4-integrins has been reported in breast cancer cell lines and in mammary tumor tissue sections {Natali 1992; Gui 1995; Zutter 1995}. These changes in integrin expression may result in altered cell surface ratios of individual integrins, which could affect tissue organization and lead to tumor progression via altered intracellular signaling. Here we show that the effect of laminin-1 on ERα levels is affected by α2, α6, and β1 integrin blocking antibodies while the effect of collagen-IV is affected by α2 and β1 integrin blocking antibodies as would be expected from what is known about these receptors and their ligands.

The α2 gene promoter contains ER binding sites (EREs) {Zutter 1994}, suggesting that ER may play a role in the regulation of integrin expression and therefore in tumor cell invasion through the BM. It is possible that collagen type IV signaling and ER are connected by a positive feedback loop in mammary epithelial cells: collagen type IV, through its receptor α2β1, increases ERα levels, and in turn, ERα stimulates the expression of α2 integrin subunit. In this regard, ER gene expression has been positively correlated with α2β1 integrin and collagen-IV expression in breast carcinomas. Ductal carcinomas that lack ER also lack α2β1 expression and are more invasive {Maemura 1995; Lanzafame 1996}. Therefore, it seems possible that the interconnection of these pathways, ER and α2β1 integrin, are involved in breast tumor progression.

A critical determinant of breast cancer progression and therapeutic management of breast cancer patients is the hormonal responsiveness of the tumors, which correlates with ER expression in tumor cells {Lapidus 1998; Sommer and Fuqua 2001}. The presence of significant amounts of ERα in breast cancer is an indication of hormone-dependence and such tumors are often responsive to the antiestrogen tamoxifen. Loss of ER gene expression/function in breast tumors is one of the most important steps in acquiring hormone resistance, and such tumors usually display a less differentiated and more aggressive phenotype. Because inactivating genetic mutations at the ERα locus or inactivation of ER gene expression caused by CpG island methylation are unlikely to account for the large number (one third) of tumors that lack ER expression or function {Lapidus 1998; Murphy 1998}, the mechanisms that govern ERα expression and function have become a key focus of research in breast cancer.

Conclusion

We have determined that adhesion to particular components of the BM up-regulates ERα both in primary cultures of normal mammary epithelial cells and in an established mammary epithelial cell line, but not in mammary fibroblasts. Thus, context-dependent regulation of ER activity appears to be a fundamental and specific property of mammary epithelial cells. Furthermore, these data provide a possible explanation for the loss of ER expression that occurs during breast tumor progression: as mammary epithelial cells progress to malignancy and invasion, they lose normal interactive contact with the BM {Petersen 1992; Werb 1996; Bissell and Radisky 2001}, thereby losing the signals that maintain the normal expression of ER. This model system may be used to understand why some breast cancers develop as ER-positive tumors, and some develop as more aggressive hormonal-resistant ER-negative ones.

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Legends

Figure 1. *rBM partially restores ERα protein levels in primary cultures of mammary epithelial cells, without affecting its levels in primary fibroblasts.* **A**. Morphology of (a) freshly isolated mammary organoids derived from 12-weeks old- nulliparous mammary glands from Balb/c mice (time 0); (b) primary epithelial cells grown for 2 days on plastic; or (c) on top rBM. Magnification 10x. (bar, 100 μm). **B**. Although ERα expression decreased after one day in culture, its expression was partially maintained when rBM was present. Representative western blots showing ERα and β-casein protein expression in cell extracts derived from primary mammary epithelial cells grown for 10 days on plastic (a) or on top of rBM (b). E-cadherin was used to normalize the values of ERα. Quantification by densitometry (c) of ERα protein levels from mammary epithelial cells grown on plastic (solid line) or on top of rBM (dotted line). ERα was expressed in relative units to protein levels at time 0 (day of isolation of the organoids that corresponds to value = 1). Replicates=5 (five different primary culture preparations using a pool of bilateral 4th inguinal mammary glands from 10 mice in each preparation. *:$p<0.05$ vs. plastic according to Student t-test. **C**. Morphology of mammary fibroblasts grown for 6 days on plastic (a) or on top of rBM (b). Magnification 10x. (bar, 100 μm). **D**. ERα protein expression in epithelial cells (ep) grown for 2 days on plastic and in fibroblasts grown for the same period of time on plastic (pl) or on top of rBM (rBM). ERα was not regulated by rBM in primary fibroblasts.

Figure 2. *rBM increases ERα protein levels in a clonal mammary epithelial cell line.* **A**. Morphological cell changes in an epithelial cell line, Scp2. Cells grown on plastic formed a flattened monolayer (a), whereas cells grown on top of rBM aggregated and formed acinus-like structures (b), magnification 20x (bar, 100 μm). They progressively acquire a morphological change (rounding) when cultured in the presence of increasing concentrations of rBM (c,d,e), magnification 10x (bar, 100 μm). **B**. ERα protein levels in cell lysates were determined by western blot (a), quantified by densitometry and they were expressed relative to the values measured on plastic (b). Replicates n=5. *:$p<0.05$ vs. plastic. **C**. The proportion of ERα-positive cells was higher in the presence of rBM. Immunocytochemistry of ERα in Scp2 cells grown on plastic (a,b) or in the presence of 2% rBM (c,d). Note that not all the cells (DAPI nuclear; blue) stained for ERα (green), and that the staining for ERα was predominantly nuclear. Magnification 60x. (bar, 20 μm). The table shows mean +/- standard deviation corresponding to (e) percentages of ERα-positive cells and to (f) ERα expression levels quantified in single cells grown on plastic or in the presence of rBM. Intensity of fluorescence was expressed as arbitrary units (A.U.). At least 100 cells were analyzed for each culture condition.

Figure 3. *rBM increases an ER-mediated response independently of estradiol.* **A**. Morphological cell change in Scp2-ERE-TK-CAT cells grown on plastic (a) or on top rBM (b). Magnification 40x (bar 25 μm). **B**. ER-mediated transcriptional activity (measured as CAT reporter gene activity) was quantified in cells grown on platic or on top rBM, in the presence or absence of 17β-estradiol 10^{-8}M, or the antagonist ICI 182,780 10^{-7}M for 48 hr in culture. Replicates=4. *:$p<0.01$ vs. control (vehicle ethanol).

Figure 4. *rBM increases ERα mRNA levels in mammary epithelial cells.* ERα mRNA was analyzed by quantitative RT-PCR (A) 2 and 6 days after isolating primary cells from the mammary gland and culturing them on plastic or in the presence of rBM (dripped or on top), or (B) after 4 days in culture for Scp2 cells. Values were normalized with respect to GAPDH mRNA. Three independent experiments comprising different primary culture preparations or Scp2 cultures were used to create the graph. *: $p < 0.05$ vs. plastic for each condition.

Figure 5. *The regulatory effect of rBM on ERα expression is not due to changes in growth rate.* **A**. Proliferation of Scp2 cells grown on plastic (a, b, c) or in the presence of rBM (a, d, e) after adding different concentrations of insulin (in the absence of serum or other growth factor). Proliferation was determined by BrdU labeling followed by immunofluorescence. Images are shown only for 0 (b,d) and 10 μg/ml insulin (c,e), where cells that are positive for BrdU appear cyan after overlying with the nuclear staining performed with DAPI (blue). Magnification 20x. **B**. ERα protein levels in Scp2 cells grown under

different cell proliferation status indicated in A were evaluated by western blot (a, representative gel) and quantified by densitometry (b). Replicates n=4. *:p<0.05 vs. no insulin.

Figure 6. *The regulatory effect of rBM on ERα levels is independent of cell shape changes.* **A.** Primary cells (a) and Scp2 cells (b) form typical flattened monolayers on plastic. Morphological change (cell rounding and aggregation) can be induced by the non-adherent substratum polyHEMA in primary cells (c) and in Scp2 cells (d). The addition of rBM on polyHEMA did not affect this cell rounding (e,f). **B.** The densitometric analysis of ERα protein levels determined by western blot in primary mammary epithelial cells at days 2 or 6 after isolation (a) and in Scp2 cells at day 4 in culture (b) shows that addition of rBM to pre-rounded cells was necessary to up-regulate ERα. Replicates n=3. *:p<0.05 vs. plastic. Magnification 10x (bar 100 μm).

Figure 7. *Collagen-IV and laminin-1 are the BM components responsible for the regulatory effect on ERα levels in mammary epithelial cells.* **A**. Isolated mouse mammary epithelial organoids (a) were grown in the presence of different matrix components dripped into the medium: collagen-I (b), collagen-IV (c), laminin-1 (d) or a mixture of laminin-1 and collagen-IV (e). In all cases the organoids were attached and spread on the plastic after 2 days in culture (bar, 100 μm). ERα protein levels were measured in these different culture conditions by western blot at days 2 and 6 after culture, and expressed as relative units to levels at time 0 (f). Replicates = 4 (4 different primary culture preparations). *:p<0.05 vs. plastic. **B**. Scp2 cells formed monolayers after dripping collagen-I (a) or collagen-IV (b), and adopted a rounded morphology when laminin-1 was dripped to the plastic (c) (bar, 100 μm). ERα protein levels were determined by western blot, quantified by densitometry and they were expressed relative to the values measured on plastic (d). Replicates n=5. *:p<0.05 vs. plastic.

Figure 8. *α2, α6 and β1 integrin subunits mediate the regulatory effect of collagen-IV and laminin-1 on ERα levels.* ERα protein levels were determined by western blot in primary mammary epithelial cells (A) and Scp2 cells (B) after adding mouse IgG (control, c), or 10 μg/ml of α1, α2 or α6 or 5 μg/ml of β1 integrin blocking antibodies to cells grown on plastic or in the presence of collagen-IV, laminin-1 or rBM. Replicates=3. *:p<0.05 vs. control (IgG addition).

Materials and Methods

Antibodies and Reagents

The function-blocking integrin antibodies against α1 (Ha31/8), α2 (HMα2), α6 (GoH3) and β1 (Ha2/5) subunits were purchased as sodium azide- and endotoxin-free reagents from PharMingen (San Diego, CA). The anti-ERα polyclonal antibody (MC-20) purchased from Santa Cruz (Santa Cruz, CA) was used for western blots. The anti-ERα monoclonal antibody (NCL-ER-6F11) purchased from NovoCastra (Newcastle, United Kingdom) was used for immunostaining. The monoclonal anti-rat β-casein antibody was a gift from Dr. C. Kaetzel (Case Western Reserve University, Cleveland, OH). The anti-E-cadherin antibody (C20820) was purchased from Transduction Laboratories (Lexington, KY). rBM (Matrigel), lamimin-1 (entactin-free), collagen-IV and MatriSperse Cell Release Solution were purchased from Collaborative Biomedical Products (Bedford, MA). Collagen-I was purchased from Vitrogen (Palo Alto, CA). poly(2-hydroxyethyl methacrylate) (polyHEMA) was purchased from Sigma Chemical. Collagenase type A and 5-Bromo-2'-deoxy-uridine (BRdU) were purchased from Boehringer-Mannheim (Indianapolis, IN). Alamar blue was purchased from (Accumed International, Westlake, OH).

Cell culture

Primary cultures: Primary mammary epithelial cells were obtained by a procedure slightly modified from {Kittrell 1992/d}. Briefly, after removal of the 4th inguinal mammary glands from nulliparous 12- week-old virgin BALB/c mice, they were minced by chopping with two razor blades in parallel. Mammary cells were dissociated by collagenase type A (2 mg/ml) in the presence of 5 μg/ml insulin (Sigma Chemical) and with antibiotics (600 U/ml of nystatin (Sigma Chemical), 100 U/ml of penicillin-streptomycin and 50 μg/ml gentamycin (Gibco)) in DMEM/F12 medium for 3 hours at 37°C with constant shaking (100 rpm). The resulting suspension was centrifuged at 1000 g for 10 min., and the pellet resuspended in 4 ml DMEM/F12 containing 2 U/ml DNase (Sigma Chemical). After gently shaking for 2 min. the DNase was diluted by adding 4% fetal bovine serum (FBS) in 4 ml of DMEM/F12 medium, and the final suspension (containing 2% FBS in DMEM/F12) was centrifuged again at 1000 g for 10 min. The resulting pellet is resuspended in phosphate-buffered saline (PBS) containing 5% adult bovine serum (Atlanta Biologicals, Norcross, GA), and this procedure was repeated 6 times to remove fibroblasts, with short pulses of spinning at 1500 g. This protocol yielded 90% or greater purity of epithelial cells (mostly as organoids of approximately 100 cells), as determined by keratin and vimentin immunocytochemistry after cell dissociation with trypsin (data not shown). Each fraction, pellet (epithelial cells) and supernatant (fibroblast cells), was resuspended in growth medium (indicated below). The day of the isolation from the gland is considered time 0 in the culture period.

Cell lines: Scp2 is a functionally normal mouse mammary epithelial cell line established in our laboratory {Desprez 1993}. The Scp2-ERE-TK-CAT cell line is a derivative of Scp2 that have been stably transfected by cotransfecting 30 μg of the pA2(-331/-87)tk-CAT8+ plasmid and 3 μg of pSV2neo plasmid. pA2(-331/-87)tk-CAT8+ contains the chloramphenicol acetyltransferase (CAT) enzyme as a reporter gene, under the control of a minimal thymidine kinase (TK) promoter containing an upstream consensus estrogen-response element (ERE). The ERE corresponds to the region –331 to –87 of the Xenopus vitellogenin A2 gene {Klein-Hitpass 1986}. The resulting SCp2-ERE-TK-CAT cells were obtained by pooling neomycin resistant colonies. They were selected under 400 μg/ml G418 (Gibco, Rockville, MA) and maintained under 40 μg/ml G418. They were used at passage 6-8 after transfection/selection.

Scp2, Scp2-ERE-TK-CAT, and primary mammary cells were cultured at a density of ~50,000 cells/cm^2 or ~100,000 cells/cm^2 (in the case of cultures on top rBM and on polyHEMA, see below) in DMEM/F12 medium containing 50 μg/ml gentamycin, 5 μg/ml of insulin, 1 μg/ml of hydrocortisone and 3 μg/ml of prolactin (Sigma Chemical). For primary cultures, the growth medium was supplemented with epidermal growth factor (EGF, 5 ng/ml), linoleic acid (5 μg/ml; Sigma) and bovine serum albumin (BSA, 5 mg/ml; Sigma). Attachment and spreading of the cells to the covered-glass chamber slide (for immunocytochemistry) or the plastic dish were performed for 24 hours of culture in the presence of 2% FBS. After that, the cells were grown for the days indicated in each case with fresh serum-free medium

containing insulin, hydrocortisone and prolactin, with or without addition of ECM components (see below). In experiments where the ER activity was measured (CAT reporter assays) we used charcoal-treated FBS (HyClone, Logan, Utah) and phenol red free DMEM/F12 medium to avoid interference from exogenous estrogens. When indicated, 10^{-8}M of 17β-estradiol (Sigma Chemical), 10^{-7}M of the antagonist ICI 182,780 (Tocris Cookson, Ellisville, MO), or the same volume of ethanol (vehicle) were added to the medium for the last 48 hours.

Culture substrata

The culture conditions for attached cell lines or primary cells consisted of untreated tissue culture plastic or plastic covered by a thick layer (50 μl/cm^2) of a growth factor reduced reconstituted basement membrane (rBM) derived from Englebreth-Holm-Swarm tumor (Matrigel) and the cells were seeded on top of the gel (on top rBM). Matrigel was previously allowed to solidify at 37°C for 40 min. For assays in pre-rounded cells, primary or Scp2 cells were cultured in suspension by placing ~100,000 cells/cm^2 in a culture dish coated with the nonadhesive substratum poly(2-hydroxyethyl methacrylate) (polyHEMA) in the corresponding serum-free medium (see above). PolyHEMA-coated dishes were prepared using a solution of 6 mg/ml polyHEMA in 95% ethanol added to culture plates at 0.05 ml/ cm^2 and allowed to evaporate to dryness.

For the "dripping" conditions, soluble rBM, or purified ECM components laminin-1, collagen-I, collagen-IV or fibronectin were diluted in the culture medium, and were added as an overlay to previously attached and spread cells in the case of Scp2 cells or to primary cultures immediately after isolation from the gland. In the case of polyHEMA cultures, when indicated, rBM was mixed in the medium with the cells. For rBM we tested 1, 2 and 5% dilution from a 10 mg/ml protein concentration of Matrigel. Since the most effective dilution was 2%, we estimated the final concentration for the ECM components corresponding to their relative proportion in 2% Matrigel. The final concentrations used were: 150 μg/ml of laminin-1, 20 μg/ml of collagen-I, 20 μg/ml of collagen-IV, and/or 10 μg/ml fibronectin. In these conditions, the components form a precipitate covering the cultured cells.

Cellular lysis

Cells were treated for the indicated days (see particular cases), with one change of medium every two days, and at the end of the culture period, cells were lysed and extracted for protein or RNA analysis. For lysis and extraction, cells were rinsed once with PBS and after that, either protein extraction using the protein extraction reagent for mammalian cells (M-PER; Pierce, Rockford, IL) or total RNA extraction using the RNeasy Mini kit (Qiagen, Valencia, CA) were performed following manufacturer's directions. For cells growing on top of rBM, before the cell lysis, the cells were removed from the gel by using enzymatic digestion with MatriSperse for 1 hour on ice, followed by a centrifugation for 5 min. at 1000 g. The resulting pellet was then subjected to protein or RNA extraction as described above. For cells growing on polyHEMA-coated dishes, they were transferred to Eppendorf tubes, centrifuged and lysed as described above.

Integrin blocking

Scp2 cells or primary mammary epithelial cells were grown on plastic or in the presence of rBM, collagen-IV, or laminin-1, for 4 days in the presence of 10 μg/ml of mouse IgG (control, c) or in the presence of 10 μg/ml of α1, α2 or α6 or 5 μg/ml of β1 integrin blocking antibodies. The antibodies were diluted in the corresponding medium at the time of plating the cells on top rBM, or after 24 h of plating them on any other condition to let them attach and spread. At the end of the experiment, cell lysis followed by protein extraction was performed as described above. Cell viability using Alamar blue vital dye assay was carried out in parallel cultures according to manufacturer's instructions.

Immunocytochemistry for ERα

For immunocytochemistry, cells were fixed with –20°C methanol:acetone (1:1) solution for 5 min., air dried for 10 min., rehydrated in PBS, blocked with Super Block Blocking Buffer in PBS (Pierce) and incubated with ERα monoclonal antibody (NCL-ER-6F11) followed by FITC-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA), mounted and observed under fluorescence microscopy. Before mounting, 4',6-Diamidino-2-Phenylindole (DAPI, Sigma) was used to stain DNA. Control experiments were carried out omitting the primary antibody. Images were captured using Spot RT camera and software (Technical Instruments, Burlingame, CA). Cellular labeling indices for ERα were determined by counting at least 100 cells from randomly selected visual fields and calculating the intensity of labeling in the cells by using Simple PCI imaging software (Compix, Inc. Imaging Systems, Cranberry Township, PA).

Cell proliferation assay

To study the influence of cell proliferation on ERα expression, Scp2 cells cultured for 3 days on plastic or in the presence of rBM were treated with increasing amounts of insulin: 0, 0.25, 5 or 10 μg/ml. The cells were maintained for another 24 h, including a 6-h labeling period with 10 μM BrdU (BrdU Labeling and Detection kit to measure DNA synthesis) according to the manufacturer's instructions. Nuclear labeling indices were determined by counting at least 100 cells from randomly selected visual fields and calculating the percentage of cells with labeled nuclei. Parallel experiments were performed to extract proteins and detect ERα levels by western blot.

CAT assay

We used the nonradioactive FLASH CAT Assay kit (Stratagene, La Jolla CA) to measure the CAT activity in cell lysates from Scp2-ERE-TK-CAT cells. Briefly, we mixed 5 μg protein/sample quantified by protein assay (Protein Assay DC, Bio Rad, Hercules, CA) with the fluorescent derivative of chloramphenicol BODIPY (borondipyrromethane difluoride fluorophore). This substrate is converted to a single monoacetylated product by CAT that is separated from the substrate by thin layer chromatography (TLC). The TLC plates were scanned using STORM fluoroimager (Amersham Biosciences, Sunnyvale, CA) and quantitation was performed using ImageQuant (Amersham).

Western blot for ERα and β-casein detection

Equal amounts of protein (20 μg of cellular extracts) were treated with reducing protein sample buffer and were size-fractionated in a 10 % SDS-PAGE gel. The resulting gel slabs were electrotransferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH), and the membranes incubated for 2 hr at room temperature in blocking buffer containing 5% non-fat milk in 0.1% Tween-PBS pH 7.5. The blots were incubated with specific primary antibodies for 1 hr at room temperature. The antibody used for loading control recognizes the 120-kDa transmembrane glycoprotein E-cadherin. To detect ERα, we used a polyclonal antibody raised against the C-terminus of the protein, MC-20 (Santa Cruz) which recognizes a band of ~67 kDa. The monoclonal antibody used to detect β-casein recognizes a band of ~30 kDa. The blots were washed appropriately with 0.1% Tween-PBS followed by the addition of the appropriate horseradish peroxidase-conjugated secondary antibody. After 1 hr of incubation and appropriately washes, the signal was detected using the SuperSignal West Dura detection kit (Pierce). The intensity of each band was quantified using the ChemiImager (Alpha Innotech Corporation, San Leandro, CA) scanning densitometry equipment.

Quantitative PCR

cDNA was prepared with 2 μg of total RNA using M-MLV Reverse Transcriptase and oligo-dT primer (Gibco Life Technologies, Gaithersburg, MD) according to manufacturer instructions. Quantification was done using LightCycler and the DNA Master Syber Green I kit (Roche, Indianapolis, IN). The set of primers used in the PCR (forward primer 5' AGACCGCCGAGGAGGGAGAATGTT 3' and reverse primer 5' GGAGCGCCAGACGAGACCAATC 3') amplify the region between +783 and

+1197 of ERα mRNA corresponding to the C-terminus of the protein. To normalize the values of ERα we performed a quantification of GAPDH (forward primer 5' CCCCTGGCCAAGGTCATCCATGAC 3' and reverse primer 5' CATACCAGGAAATGAGCTTGACAAAG 3') in the same samples. The reactions for both amplifications were carried out for 40 cycles with an annealing temperature of 59°C.

Statistics

All values presented in this study are means +/- s.e.m. of at least three independent experiments. Comparisons between groups were performed employing one-way analysis of variance, and differences between means were determined by a Student-Newman-Keuls multiple comparison test.

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